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Distinct Role of Follicular Dendritic Cells and T Cells in the Proliferation, Differentiation, and Apoptosis of a Centroblast Cell Line, L3055[1]

Jongseon Choe,[†] Li Li,[*] Xin Zhang,[*] Christopher D. Gregory,[‡] and Yong Sung Choi[2*]

Germinal center (GC) B cells undergo complex interactions with follicular dendritic cells (FDC) and T cells in the course of differentiation into memory B and plasma cells. To delineate the individual roles of FDC and T cells at each stage of GC B cell differentiation at the clonal level and to analyze the signals involved, we adopted a unique experimental model using an FDC line, HK, and a lymphoma cell line, L3055, that resembles centroblasts. A detailed phenotypic analysis revealed L3055 cells to be a clonal population originating from the GC. Like freshly isolated centroblasts, L3055 cells underwent spontaneous apoptosis when cultured in the absence of fresh FDC or HK cells. L3055 cells proliferated continuously in the presence of HK cells, while they differentiated into a population with the phenotype of centrocytes after stimulation with CD40 ligand (CD40L) and IL-4. The CD40L-stimulated L3055 cells underwent CD95-mediated apoptosis, which was reminiscent of the feature of CD40L-stimulated tonsillar GC B cells. In contrast to HK cells that did not protect L3055 cells from anti-Ig killing, CD40L plus IL-2, IL-4, and IL-10 prevented anti-Ig-induced apoptosis. These experimental results demonstrate a distinct function of FDC and activated T cells, in that FDC provide signals for rapid proliferation of centroblasts, whereas T cells confer signals for differentiation of centroblasts into centrocytes and resistance to B cell receptor-mediated apoptosis. T cells collaborate with FDC in the protection and expansion of the Ag-specific GC B cells. *The Journal of Immunology,* **2000, 164: 56–63.**

The germinal center (GC)[3] of secondary lymphoid follicles provides a microenvironment for B cells to undergo clonal expansion and selection before differentiating into memory B cells (1). The GC reaction is initiated by rapid proliferation of few Ag-stimulated B cells in association with follicular dendritic cells (FDC) (2). The mechanism for this rapid growth is largely unknown, although the low threshold for cellular activation was suggested (3). The GC B cells exhibit features distinct from those of naive or memory B cells, in that they display a unique pattern of Ag expression on the cell surface (4), undergo Ag receptor-mediated apoptosis (5), and require essential survival signals from FDC because disruption of FDC-B cell clusters results in apoptosis of B cells (6–8). This *in vitro* observation was further confirmed *in vivo* by demonstrating in the lymphotoxin-α knockout mice that the initial interaction between FDC and B cells is essential for GC formation (9, 10). T cells expressing CD40 ligand (CD40L) at the same time play a pivotal role in the GC reaction, as evidenced in hyper-IgM patients (11) and in mouse models that are deficient for CD40 (12) or CD40L (13, 14). The signals for survival, proliferation, and differentiation of GC B cells, however, are poorly understood, in part due to the lack of a proper *in vitro* model to clearly analyze the cellular and molecular interactions between B cells and FDC. Furthermore, the identification of molecular signals that induce somatic mutation and class switching has been hampered by the low viability of GC B cells under conventional culture conditions without FDC and the polyclonality of freshly isolated GC B cells.

To overcome the practical difficulty in isolating pure FDC and to mimic the GC reaction *in vitro*, we have established an FDC line, HK, from human tonsils and used it to determine molecular and cellular requirements for GC B cells (15–19). HK cells indeed have functional features of FDC in delaying apoptosis and stimulating growth and differentiation of GC B cells. HK cells bind and prevent apoptosis of $IgD^{-}CD38^{+}CD44^{-}$ GC B cells preferentially and have costimulatory effects on the proliferation of CD40-stimulated GC B cells (16, 17). However, the individual roles of HK cells and CD40L in the proliferation and differentiation of GC B cells were not clearly defined, since both were required for optimum growth. In addition, GC B cells freshly isolated from tonsil are heterogeneous with regard to the stage of differentiation, mutation frequency, and Ig class (20) and are not ideal for characterizing the external signals operating at each stage of B cell differentiation in the GC. A monoclonal population of dividing cells would be devoid of such problems of freshly isolated GC B cells.

Burkitt's lymphoma (BL) is a tumor with features of GC B cells (21), and group I BL lines retain those features *in vitro*. Gregory et al. (22) suggested the GC origin of BL cells by demonstrating the association with GC B cell-specific surface markers, CD10 and CD77. The BL cells display a homogeneous cell surface phenotype and are inclined to undergo spontaneous apoptosis unless cultured with appropriate stromal cells (23). In the present study we have chosen an EBV-negative group I BL cell line, L3055 (24, 25), to investigate molecular and cellular signals for the survival, proliferation, and differentiation of GC B cells at the clonal level. We

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[3] Abbreviations used in this paper: GC, germinal center; FDC, follicular dendritic cells; BL, Burkitt's lymphoma; CD40L, CD40 ligand; PI, propidium iodide; BCR, B cell receptor.

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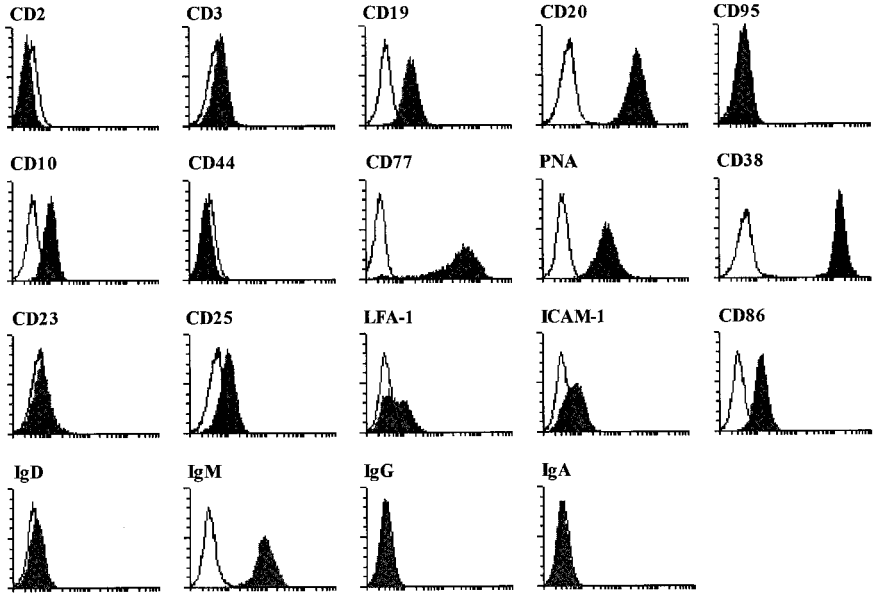


FIGURE 1. Flow cytometric analysis of L3055 cells reveals the phenotype of centroblasts. Blank histograms represent controls stained with isotype-matched Ab.

show that L3055 cells express typical surface markers of centroblasts, exhibit a high propensity to undergo spontaneous apoptosis, but continuously proliferate without differentiation when cocultured with FDC clusters or HK cells. The addition of CD40L and IL-4 induces differentiation from centroblastic to centrocytic phenotype, and L3055 cells with the latter phenotype undergo CD95-mediated apoptosis. HK cells expand the L3055 cells rescued from B cell receptor (BCR)-mediated apoptosis by CD40L and a cytokine mixture (IL-2, IL-4, and IL-10). These data suggest important roles for FDC and T cells in the proliferation, differentiation, and selection of B cells in the GC.

Materials and Methods

Cell lines

The L3055 cell line was isolated from an HIV-negative 17-yr-old male of Arabic origin with an initial diagnosis of Burkitt-type acute lymphoblastic leukemia (24, 25). We subcloned L3055 cells by screening for the property of being HK dependent. L3055 cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (Life Technologies, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Irvine Scientific). HK cells were established and maintained as described previously (15).

Antibodies and reagents used in the study

FITC- or PE-conjugated anti-CD10 (W8E7), anti-CD23 (EBVCS-5), anti-CD23 (S5.2), anti-CD3 (SK7), anti-CD19 (4G7), anti-CD25 (2A3), anti-CD20 (L27), anti-CD38 (HB-7), goat anti-mouse Ig, and isotype controls were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated anti-CD44 (F10-44-2) was obtained from BioSource International (Camarillo, CA); anti-CD86 (HA3.1F9) was obtained was from Genetics Institute (Cambridge, MA); FITC-conjugated anti-IgD (HJ9) was obtained from Sigma (St. Louis, MO); biotin-conjugated peanut agglutinin was obtained from Vector Laboratories (Burlingame, CA); FITC-labeled anti-CD95 (DX2), biotin-labeled anti-IgG (G18-145), and biotin-labeled anti-IgA (G20-359) were purchased from PharMingen (San Diego, CA); FITC- or PE-labeled streptavidin and anti-CD95 (CH-11) were obtained from Immunotech (Westbrook, ME); FITC-conjugated goat anti-mouse IgM was purchased from Southern Biotechnology Associates (Birmingham, AL); anti-IgM (SADA4.4) was obtained from American Type Culture Collection (Manassas, VA); the annexin V-FITC apoptosis detection kit was obtained from Trevigen (Gaithersburg, MD); and Transwell (0.4-μm pore size) was purchased from Corning Costar (Cambridge, MA). Anti-CD77 (5B5) was provided by Dr. M. Nahm (University of Rochester, Rochester, NY). Soluble human trimeric CD40L was provided by Dr. R. Armitage (Immunex, Seattle, WA) and used at 400 ng/ml, which was determined to give optimal proliferation of GC B cells. The IL-2 was obtained from Hoffmann-La Roche (Nutley, NJ). Recombinant human IL-4 was a gift from Schering-Plough (Union, NJ). The IL-10 was purchased from R&D Systems (Minneapolis, MN).

Isolation of FDC clusters

Fresh FDC clusters were isolated as described by Bosseloir et al. (26). After mincing the tonsil tissue into small pieces, an enzyme mixture of collagenase IV (2 mg/ml; Worthington, Freehold, NJ) and DNase (2 U/ml; Sigma) was used to digest the tissue three times at 37°C, 20 min each time. The cell suspension was separated by a Percoll density gradient with 0, 15, 35, 50, and 60% Percoll. The interface of 15–35% was collected and washed. A BSA density gradient of 0, 3, and 7.5% was then used to further purify fresh FDC clusters by centrifuging at $10 \times g$ for 10 min at 4°C. The 7.5% phase containing FDC clusters was collected and used in the experiments.

Flow cytometry

Flow cytometric analysis was conducted on a FACScan (Becton Dickinson) with CellQuest software as described previously (17). The computer software FACSComp calibrated the cytometer with CaliBRITE beads (Becton Dickinson).

Coculture

HK cells irradiated with 3000 rad were prepared 1 day before L3055 cells were added to the wells of 24-multiwell plate. Coculture was performed under various conditions as indicated in the figures. A cytokine mixture of optimal concentrations of IL-2 (20 U/ml), IL-4 (100 U/ml), and IL-10 (50 ng/ml) was used throughout the experiments. Optimal concentrations of anti-IgM (10 μg/ml) and anti-CD95 (100 ng/ml) were used. Viable cells were enumerated by counting the cells with intact morphology after staining with trypan blue.

Statistical analysis

The statistical significance of differences was determined using Student's t test, and $p < 0.05$ was considered significantly different.

Results

L3055 is a cell line with phenotypic and functional features of centroblasts

To study molecular signals that operate at each stage of GC B cell differentiation at the clonal level, it is necessary to determine whether L3055 cells represent centroblasts. A detailed flow cytometric analysis revealed that L3055 cells expressed CD10, CD20, CD38, CD77, and peanut agglutinin binding, but not CD44 (Fig. 1), extending the results reported previously (27). These are the typical surface markers for GC centroblasts (18, 28). L3055 cells

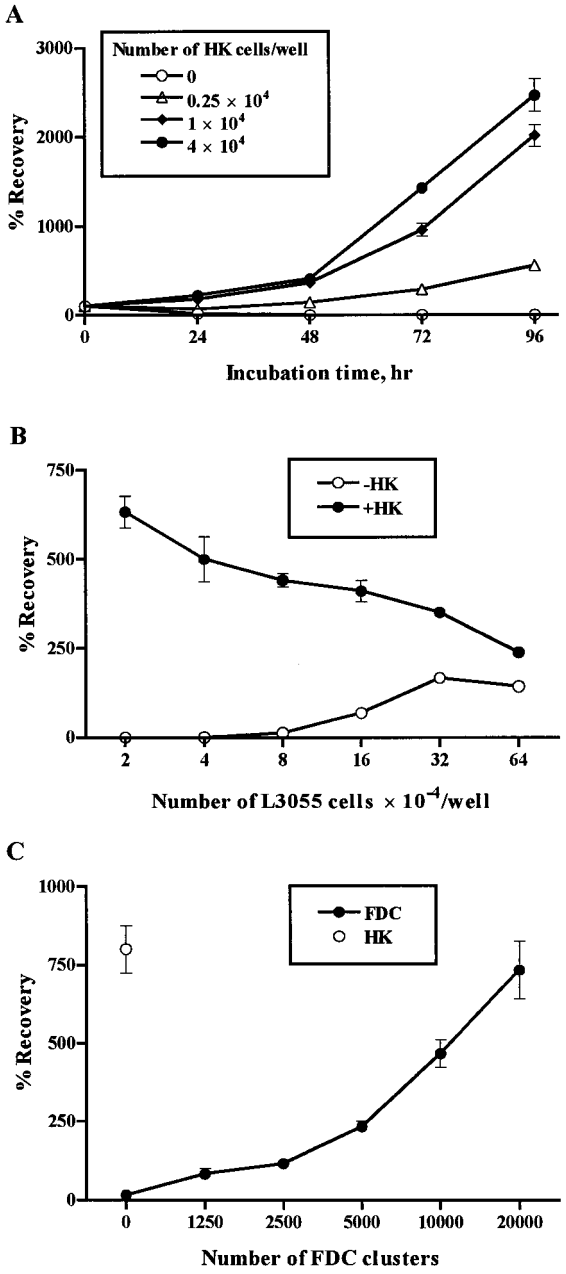
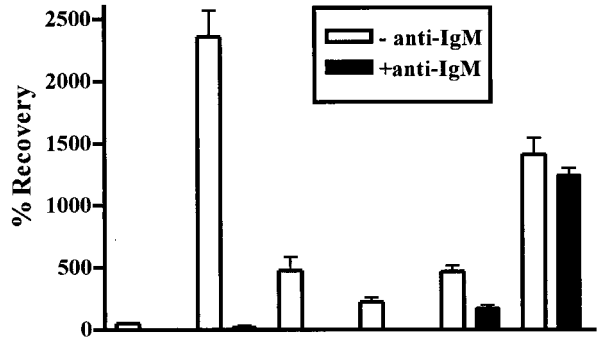


FIGURE 2. L3055 cells depend on HK cells and FDC for survival and growth. *A*, L3055 cells (2×10^4) were cultured with different numbers of HK cells, and the viable cells were counted at the indicated time points. *B*, Graduated numbers of L3055 cells were cultured in the presence of fixed number of HK cells (1×10^4) for 3 days. *C*, L3055 cells (2×10^4) were cultured for 4 days with either HK cells (2×10^4) or graduated numbers of FDC clusters. Recovery was calculated at each time point as a percentage of the initial number of viable cells. Data are presented as the mean of triplicate determinations and SEM. A representative of three reproducible results is shown.



HK	-	+	-	-	-	+
CD40L	-	-	+	-	+	+
IL-2,IL-4,IL-10	-	-	-	+	+	+

FIGURE 3. HK cells and CD40L exhibit distinct roles in the growth of L3055 cells and their response to anti-IgM. L3055 cells were cultured under the indicated conditions in the presence or the absence of anti-IgM for 4 days. Recovery was calculated as a percentage of the initial number of viable cells. The results from two independent experiments were pooled, and the mean and SEM are presented.

did not express surface Igs other than IgM. This phenotype remained constant for >1 yr in culture, indicating that this cell line did not undergo class switching to downstream isotypes.

To investigate the effect of FDC on growth, L3055 cells were cultured in the presence and the absence of an FDC line, HK. L3055 cells did not grow, and almost no viable cells were recovered in the absence of HK cells. HK cells supported the growth of L3055 cells in a dose-dependent manner, and as shown in Fig. 2*A*, their presence resulted in a >20-fold increase in cell recovery at the optimum number of HK cells. The dependence of L3055 cells on HK cells for survival and growth was not ascribed to low cell density, because increasing the cell density did not abrogate the requirement of HK cells (Fig. 2*B*). The HK-dependent growth of L3055 cells is reminiscent of a functional feature of centroblasts isolated from tonsils (16). The FDC clusters freshly isolated from tonsil were also capable of providing survival and growth signal to L3055 cells in a dose-dependent manner (Fig. 2*C*), suggesting that the effect of HK cells on L3055 cells indeed reflected an important functional feature of FDC.

HK cells provide potent proliferation signals to L3055 cells

The GC is comprised of Ag-activated T cells as well as B cells and FDC (2). Because T cells participate in GC reactions by direct cell to cell contact and by secreting cytokines (29), we used the defined signals of activated T cells, such as CD40L, IL-2, IL-4, and IL-10, to characterize HK cell signals in the proliferation of centroblasts.

When cultured alone, L3055 cells did not grow, and viable cells were rarely detected after a 4-day culture (Fig. 3). In contrast, HK cells supported growth remarkably, yielding a 24-fold increase in cell recovery. CD40L and the cytokine mixture, either alone or in combination, yielded only a 2- to 5-fold increase in the number of viable cells. When HK cells, CD40L and the cytokines were added together, the recovery of L3055 cells increased to 14-fold. However, this value was 40% less than that obtained by the addition of HK cells alone, suggesting that CD40L and the cytokines inhibited growth of L3055 cells in the presence of HK cells.

The poor recovery of L3055 cells in the absence of HK cells may have been caused by either spontaneous apoptosis or growth arrest. To distinguish between these possibilities, L3055 cells were cultured for a short period (6 h) under various conditions (Fig. 4). During this period of culture, the numbers of recovered cells were comparable under each condition. Cells were stained with FITC-labeled annexin V and propidium iodide (PI) and analyzed on a FACScan. Apoptotic cells lose membrane phospholipid asymmetry and bind annexin V on the surface, which is thought to be an early marker of apoptosis (30). Late apoptotic cells or dead cells do not exclude PI and become PI^+. In the absence of HK cells, 43% of the L3055 cells were annexin V^+ PI^+ and 20% became annexin

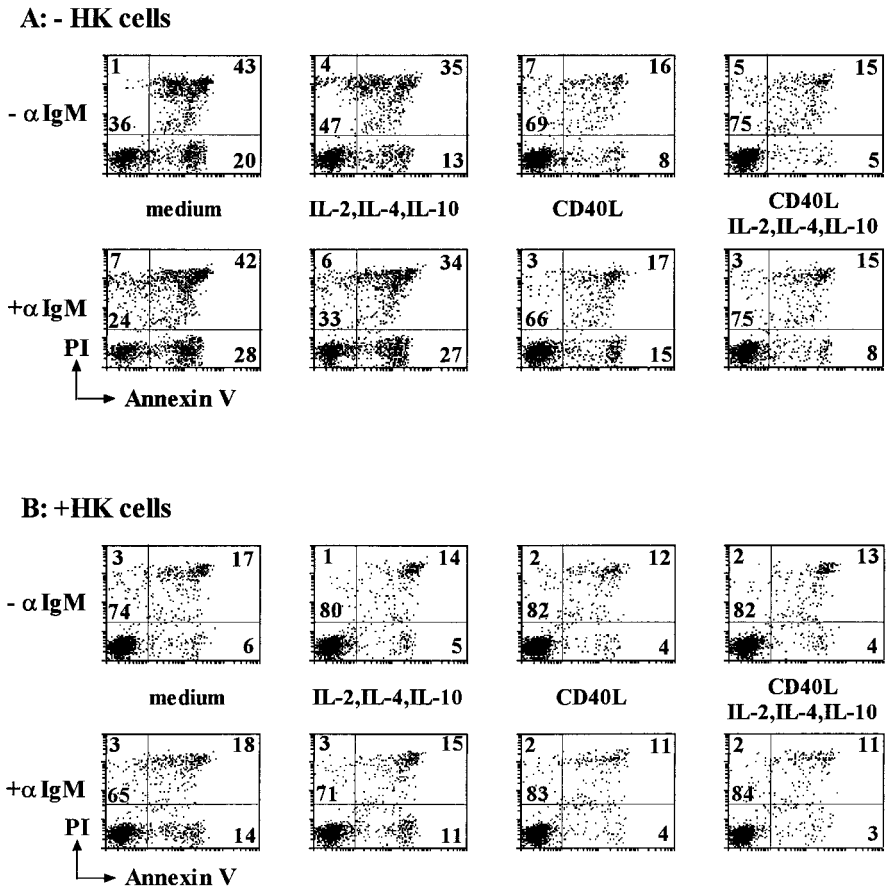


FIGURE 4. L3055 cells (1×10^5) were cultured for 6 h in the presence or the absence of anti-IgM under the indicated conditions. Cultured cells were harvested and stained with FITC-labeled annexin V and PI. Numbers represent percentages in each quadrant. These data are representative of three reproducible experiments.

V^+ PI^- (total, 63% annexin V^+). The presence of HK cells reduced annexin V^+ PI^+ cells to 17% and annexin V^+ PI^- to 6% (total, 23% annexin V^+). The addition of the cytokine mixture alone did not change the results significantly, but the addition of CD40L gave rise to results similar to those observed with HK cells regardless of whether the cytokines were added. Annexin V^- PI^- viable cells were 74% in the presence of HK cells and 75% in the presence of CD40L plus the cytokines (the *upper panels* of Fig. 4, *A* and *B*). These results suggest that CD40L and HK cells prevent spontaneous apoptosis of L3055 cells. However, CD40L plus cytokines poorly supported cellular proliferation, because the cell recovery was only 20% of that with HK cells alone (Fig. 3). These data indicate that HK cells provide signals both to prevent spontaneous apoptosis and to support cellular proliferation, whereas CD40L prevents spontaneous apoptosis but poorly stimulates the growth.

Anti-IgM-induced apoptosis of L3055 cells is prevented by CD40L and cytokines, but not by HK cells

In vivo administration of soluble Ags induces significant apoptosis of B cells in the GC (31, 32), even though Ags presented by FDC are thought to select B cells with high affinity receptors. However, the functional roles of BCR signal, FDC, and T cells in determining the fate of GC B cells have not been defined clearly. To analyze the roles of the individual factors in Ag-induced apoptosis and selection in the GC, the effect of anti-IgM on L3055 cells was examined under various culture conditions containing HK cells, CD40L, or the cytokine mixture.

No viable L3055 cells were recovered after a 4-day culture containing both anti-IgM and HK cells (Fig. 3), indicating that L3055 cells underwent anti-IgM-induced apoptosis, and HK cells did not prevent it. There was a significant recovery of L3055 cells (465%) when CD40L and the cytokine mixture were present. The addition of anti-IgM to this culture resulted in 60% less cell recovery (175%). When HK cells were added to CD40L and the cytokine mixture, however, the cell recovery obtained in the presence of anti-IgM was comparable to that in the control culture without anti-IgM (1250 vs 1400%, respectively). To detect an early sign of apoptosis induced by anti-IgM, L3055 cells were cultured under various conditions for 6 h as described previously (Fig. 3), and two-color analysis on a FACScan was performed. The addition of anti-IgM in the absence of HK cells reduced annexin V^- PI^- viable cells from 36 to 24%, which occurred with a concomitant increase in annexin V^+ PI^- early apoptotic cells from 20 to 28% (Fig. 4*A*). This result is indicative of the early onset of anti-IgM-induced apoptosis. In contrast, CD40L plus the cytokine mixture rendered L3055 cells resistant to anti-IgM killing during the 6-h culture, as addition of anti-IgM did not reduce annexin V^- PI^- viable cells (75 to 75%). Similar results were obtained in the presence of HK cells (Fig. 4*B*). The addition of anti-IgM decreased the annexin V^- PI^- viable cells (74 to 65%) with a concomitant increase in annexin V^+ PI^- early apoptotic cells (6 to 14%). These cells became resistant to apoptosis in the culture containing CD40L and the cytokine mixture (82 vs 84%). Collectively, these results suggest that HK cells do not prevent anti-IgM-induced apoptosis but provide growth signals to L3055 cells, while CD40L plus the cytokine mixture blocks BCR-mediated apoptosis but poorly stimulates the growth. L3055 cells receive the resistance signal from CD40L plus cytokines and the growth signal from HK cells, as demonstrated in the comparable cell recoveries regardless of anti-IgM addition.

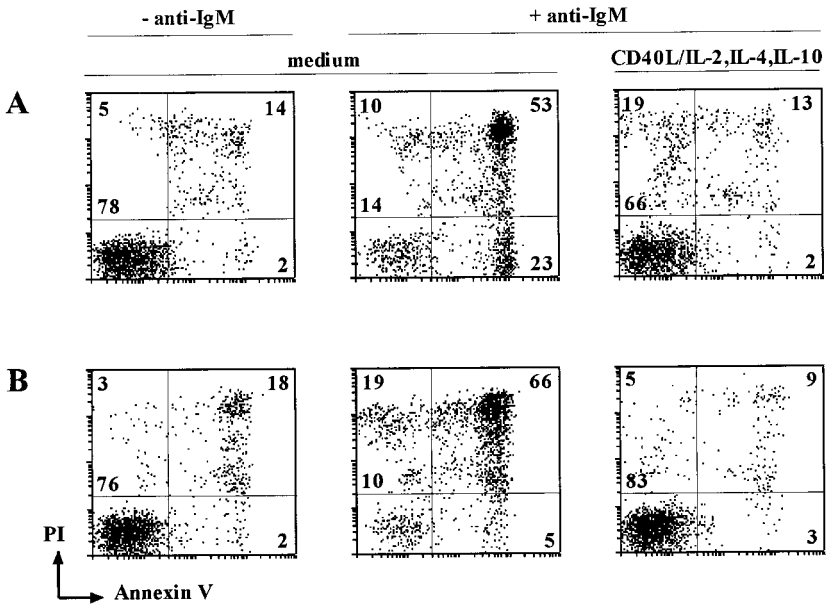


FIGURE 5. L3055 cells with centrocytic as well as centroblastic phenotype undergo BCR-mediated apoptosis. L3055 cells were cultured on HK cells in the presence (*B*) or the absence (*A*) of CD40L and the cytokine mixture for 4 days. The cultured cells under each condition were washed and split into three aliquots for an additional culture for 20 h; the first aliquot without anti-IgM, and the second and the third with anti-IgM. To examine the effect on BCR-mediated apoptosis, CD40L and the cytokine mixture were added to the third aliquot at the time of anti-IgM addition. The degree of apoptosis was measured after staining with FITC-labeled annexin V and PI. Numbers represent percentages in each quadrant. A representative of six experiments is shown.

Those cells undergoing Ag-induced apoptosis *in vivo* (31, 32) may be GC B cells that encounter Ag in the early stage of GC entrance before the encounter with Ag-activated T cells. The *in vivo* experiments did not identify whether centroblasts or centrocytes were sensitive to Ag-induced apoptosis. To investigate this question, L3055 cells were first cultured with CD40L and the cytokine mixture in the presence of HK cells for 4 days to induce differentiation and then exposed to anti-IgM for 20 h. L3055 cells cultured without CD40L and the cytokine mixture were used as a control population. Viable cells were significantly decreased in both populations by the addition of anti-IgM (78 to 14% and 76 to 10%; Fig. 5). L3055 cells cultured with CD40L and the cytokine mixture for 7 days retained the susceptibility to anti-IgM (data not shown). Both populations were protected from anti-IgM-induced apoptosis when CD40L and the cytokine mixture were present at the time of anti-IgM addition. These results suggest that both centroblasts and centrocytes are susceptible to Ag-induced apoptosis and that both populations are protected from Ag-induced apoptosis by Ag-activated T cells.

Differentiation of L3055 cells is induced by CD40L and IL-4, but not by HK cells

The order of B cell differentiation in the GC has been well characterized by surface Ag expression and localization in tonsil (33). Centroblasts in the GC differentiate to centrocytes and then to memory B or plasma cells. To determine the roles of the individual factors directing differentiation of centroblasts, phenotypic change was examined after a 4-day culture of L3055 cells under various conditions. As shown in Fig. 6, the addition of CD40L plus cytokines, particularly IL-4, resulted in a drastic change in the phenotype. The effect of CD40L or cytokines was moderate when added alone. After stimulation with CD40L and IL-4, however, CD77, the typical marker of centroblasts was down-regulated, while a marker of centrocytes, CD95, was up-regulated. Consistent with the report that BL were CD95-negative tumors (34), L3055 cells did not express CD95 before stimulation. However, the addition of IL-4, but not IL-2 and IL-10, to CD40L enhanced the induction of CD95 and CD23. CD23 is highly expressed in the light zone of the GC (35), possibly on centrocytes. Down-regulation of CD10, CD20, and CD38 was also observed in L3055 cells after culture with CD40L and IL-4 (data not shown). This result was similar to the changes observed with centroblasts freshly isolated from tonsil (18). The activity of IL-4 was specific and unique in the transition of the L3055 phenotype, because the presence or the absence of IL-2 or IL-10 did not affect the results (data not detailed). HK cells did not play a critical role in the modulation of this phenotypic change (Fig. 6).

To examine the function of CD95 induced by CD40L and IL-4, L3055 cells were precultured for 4 days with CD40L plus IL-4, and then anti-CD95 was added. These cells became sensitive to anti-CD95 and underwent apoptosis, while L3055 cells cultured without CD40L and IL-4 did not (Fig. 7). These results are in line with our previous report that CD40-stimulated tonsillar centroblasts are susceptible to anti-CD95-induced killing (17). The resistance of the unstimulated L3055 cells may be due to the absence of CD95 expression (Fig. 6). Hence, the phenotypic and functional analysis suggests that L3055 cells have the capacity to differentiate from centroblast to centrocyte in response to CD40L and IL-4. HK cells did not affect the differentiation of L3055 cells by CD40L and IL-4.

Discussion

A BL cell line, L3055, recapitulates important features of normal centroblasts. 1) L3055 cells display the phenotype of centroblasts (18); 2) they readily undergo spontaneous apoptosis, which is prevented by the FDC or FDC line, HK (16); 3) spontaneous apoptosis of L3055 cells is abrogated by CD40L and cytokines (36); 4) anti-Ig induces apoptosis of L3055 cells, which is blocked by CD40L and the cytokine mixture (27, 37); 5) CD40L and IL-4 induce differentiation of L3055 cells from the centroblastic to centrocytic phenotype (18); and 6) L3055 cells undergo CD95-mediated apoptosis following differentiation (17, 37).

For the purpose of understanding molecular signals that regulate apoptosis, proliferation, and differentiation of human GC B cells, *in vitro* experiments have been conducted by using freshly isolated GC B cells (38–40). GC B cells, which easily undergo spontaneous apoptosis, require an essential survival signal, CD40L. However, even in the presence of exogenous cytokines, CD40L poorly supports the growth of GC B cells, rendering an extensive experiment difficult (17). To solve this problem several laboratories, including ours, have successfully established FDC-like cell lines

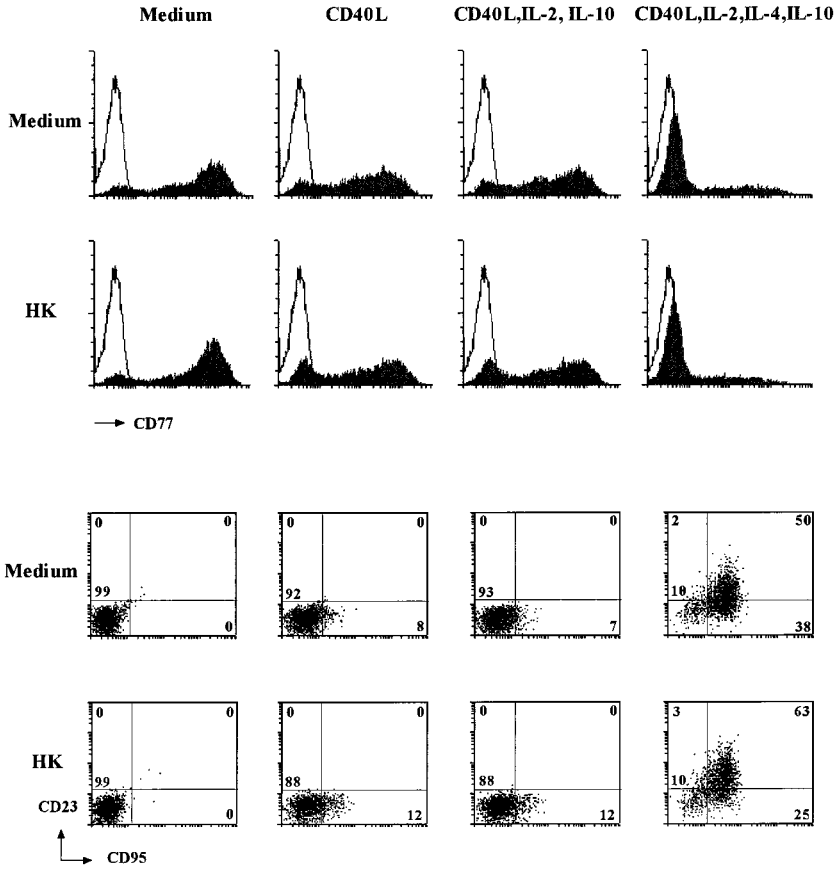


FIGURE 6. Centroblastic L3055 cells differentiate into a population with centrocytic phenotype. L3055 cells were cultured under various conditions in the presence or the absence of HK cells for 4 days. The expression levels of surface markers were measured on a flow cytometer. Blank histograms indicate controls stained with isotype-matched Ab. Numbers in dot plots represent percentages in each quadrant. The results shown are representative of five independent experiments.

(15, 41–43). For example, the presence of an FDC cell line, HK, gives rise to a >5-fold increase in cell recovery relative to that in the control culture (17). The HK cells alone do not maintain proliferation of GC B cells in the absence of T cell help. In contrast, L3055 cells continue to proliferate unaccompanied by a significant phenotypic change. This observation suggests that an important function of FDC is to maintain the proliferation of centroblasts in the dark zone, probably in the absence of T cell help. It has recently been demonstrated that the initial interactions between Ag-activated B cells and FDC are essential for GC formation in the absence of T cells (9, 10), suggesting that L3055 cells may have been derived from such early activated B cells committed to form GC. L3055 cells differentiate in response to CD40L and IL-4, indicating the distinct functions of FDC and T cells in GC formation. Centroblasts proliferate massively in the dark zone of the GC, where T cells are rare (44). In this location, activated B cells may stimulate maturation of FDC precursors via lymphotoxin-α as suggested previously (9, 10), and mature FDC, in turn, may provide proliferation signals to centroblasts. The centroblasts may differentiate into centrocytes when they encounter activated T cells. FDC appear to stimulate the survival and proliferation of centroblasts without differentiation, while T cells trigger differentiation of FDC-supported centroblasts. T cell-derived signals seem to dominate over the signals from HK cells. Thus, our data clearly delineate distinct functions of FDC and Ag-activated T cells.

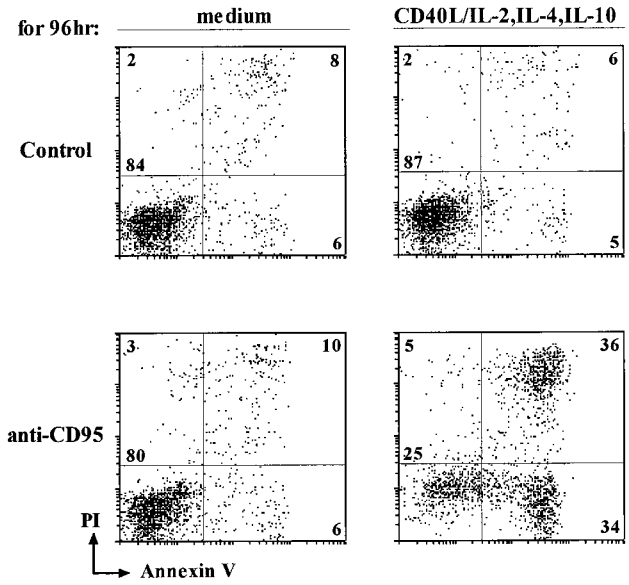


FIGURE 7. L3055 cells with centrocyte phenotype undergo CD95-mediated apoptosis. L3055 cells were cultured on HK cells under the indicated conditions for 4 days. The cultured cells under each condition were washed, split into two aliquots for further culture in the continuous presence or absence of CD40L and cytokines, and incubated for another 6 h with anti-CD95 or control Ab. The degree of apoptosis was measured by staining cells with FITC-labeled annexin V and PI. Numbers represent percentages in each quadrant. A representative of four experiments is shown.

Both centroblastic and centrocytic L3055 cells underwent anti-IgM-induced apoptosis, while only the latter was sensitive to CD95-mediated killing. Apoptosis induced by anti-IgM was efficiently prevented by CD40L and HK cells. These data underscore the complex interactions among FDC, B, T cells, and Ag in the selection and apoptosis of GC B cells. First, the data suggest that autoreactive B cells, which may arise by somatic mutation at the stage of massive proliferation of centroblasts (33), are eliminated upon the recognition of self-Ags in the microenvironment where

cognate T cells are not available, whereas Ag-specific B cells survive in the environment where cognate T cells are available. These results extend earlier observations of Holder et al. (24). Second, centrocytes also undergo apoptosis upon BCR cross-linking, because the treatment with anti-IgM induces apoptosis of L3055 cells following differentiation to centrocytes by CD40L plus IL-4. This result is in agreement with a previous report that BCR-mediated apoptosis is targeted to centrocytes (5). Although elimination of Ag-reactive GC B cells was demonstrated *in vivo* (31, 32), whether centroblasts or centrocytes were the target of Ag killing was not clear. Our findings suggest that both centroblasts and centrocytes have the propensity to undergo BCR-mediated apoptosis upon the recognition of Ag. However, Ag-specific centrocytes are protected and expanded by signals from activated T cells and FDC, respectively, as suggested by the resistance and growth of L3055 cells in the presence of HK cells, CD40L, and the cytokine mixture. Hence, our data emphasize the important function of FDC in the expansion of the Ag-specific B cells. Third, the present data suggest that CD95-mediated apoptosis operates in centrocytes, but not in centroblasts. CD95-mediated apoptosis is not operational in the early stage of the GC reaction to ensure massive proliferation of centroblasts to provide a large repertoire, but it becomes operational in the elimination of Ag-nonspecific B cells in the stage of centrocytes.

Among the cytokines produced by the GC T cells (45), the functional role of IL-4 was remarkable. The IL-4 induced differentiation of centroblastic L3055 cells into cells with centrocyte phenotype by up-regulating CD23 and CD95 and down-regulating CD10, CD20, CD38, and CD77. This is the direct effect of IL-4 on L3055 cells, as the presence or the absence of HK cells does not alter the result. These data suggest that T cells play a critical role in the differentiation of B cells in the GC by secreting IL-4 in addition to CD40L. Given the similar effects of IL-4 on L3055 cells and centroblasts freshly isolated from tonsil (18), IL-4 appears to be an essential cytokine for GC B cell differentiation. This may explain why IL-4 gene-targeted mice cannot form GC (46), and B cells in IL-4-transgenic mice display hyperactivity (47).

In conclusion, the data presented in this paper suggest that FDC (HK) and T cells (CD40L and cytokines) have distinct roles in the course of GC B cell differentiation. FDC provide unique signals for the survival and massive proliferation of centroblasts, while T cells trigger the differentiation of centroblasts and eliminate Ag-nonspecific B cells via CD95. FDC appear to cooperate with T cells in the selection step by expanding Ag-specific B cells prevented from undergoing BCR-mediated apoptosis. Further investigation with the L3055 cell line may help to elucidate molecular and biochemical events of class switching and somatic mutation.

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